In Vitro Cell. Dev. Biol.—Plant 39:573–577, November–December 2003
DOI: 10.1079/IVP2003433

1054-5476/03 $18.00+0.00

TRANSFORMATION OF SUGAR BEET CELL SUSPENSION CULTURES

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(Received 11 December 2002; accepted 26 March 2003; editor F. A. Krens)

Summary

A sugar beet transformation method was developed using particle bombardment of short-term suspension cultures of a breeding line FC607. Highly embryogenic suspension cultures derived from leaf callus were bombarded with the *uidA* (*gusA*) reporter gene under the control of either the osmotin or proteinase inhibitor II gene promoter, and the *npt* II selectable marker gene. Transient *uidA* expression was visualized as 500–4000 blue units per 200 mg of bombarded cells 2 d after bombardment. Stably-transformed calluses were recovered on both kanamycin and paromomycin media. The greatest number of GUS (+) calluses was obtained when 50 or 100 mg l^{-1} of kanamycin was applied 2 d after transformation for 3–5 wk, followed by either no selection or reduced levels of the antibiotic. PCR analyses of the GUS (+) callus lines revealed the expected size fragment for *uidA* and *npt* II genes. Stable incorporation of the *uidA* gene into the genome was confirmed by Southern blot analyses. Several transformed embryos were detected by histochemical β-glucuronidase (GUS) staining.

Key words: Beta vulgaris; biolistic; embryogenesis; *npt* II; particle bombardment; *uidA*.

Introduction

Sugar beet (*Beta vulgaris* L.) is an economically important crop that provides more than 35% of the world's sugar supply. Improvements in various sugar beet traits such as sugar yield have been achieved through conventional breeding. However, many significant agronomic problems, including the susceptibility to the fungal pathogen *Cercospora beticola* and the sugar beet root maggot, have not been solved by breeding (Bosemark, 1993; Cooke, 1993). Biotechnological approaches for sugar beet improvement have been hampered primarily by the lack of a reliable transformation method. Non-proprietary methods such as *Agrobacterium*-mediated transformation of shoot-base tissue (Lindsey and Gallois, 1990), cotyledonary node explants (Krens et al., 1996), shoot explants (Zhang et al., 2001), embryogenic callus (D'Halluin et al., 1992; Zhang et al., 2001), or polyethylene glycol-mediated transformation of guard cell protoplasts (Hall et al., 1996) are plagued by low transformation frequencies and lack of reproducibility (Snyder et al., 1999; Ivic and Smigocki, unpublished results). The particle bombardment method has proven superior for achieving truly genotype-independent transformation in agronomically important crops (Christou, 1995). However, our attempts to transform commercially important sugar beet lines using particle bombardment of hypocotyl callus (Snyder et al., 1999) that was developed with a highly regenerative tissue culture clone REL-1 (Saunders, 1998) were unsuccessful. In addition, this method entails several lengthy and labor-intensive steps for plant material preparation, including a seed germination step that is often plagued by high levels of microbial contamination.

To develop a method for transforming sugar beet breeding lines, we established highly embryogenic short-term suspension cultures from leaf callus of a breeding line that we found to be as regenerative as the tissue culture clone REL-1 (Ivic et al., 2001). Cell suspension cultures have been used successfully with the particle bombardment method to transform recalcitrant plant species (Dayton-Wilde et al., 1992; Vasil et al., 1992; Kamo et al., 1995). Use of cell suspensions for biolistic transformation is advantageous because in a relatively short time one can generate a large number of cells that, when spread as thin layers, are susceptible to transformation following bombardment with gold particles. To optimize the transformation protocol, we tested a range of selection conditions and two selection agents, kanamycin and paromomycin. We present results on transient and stable transformation of sugar beet.

Materials and Methods

Plant material and initiation of short-term cell suspensions. Sugar beet FC607 plants (Smith and Ruppel, 1980) grown in the greenhouse for 18–24 mo. were used in all experiments. Leaf discs (9 mm) were excised from expanding leaves that were surface-sterilized in 20% commercial bleach solution with 0.01% sodium dodecyl sulfate for 20 min and washed five times with sterile water. Two explants were placed in each Petri plate (100 × 15 mm) with 35 ml of B1 medium consisting of mineral salts (MS; Murashige and Skoog, 1962), 3% sucrose, 100 mg l^{-1} *myo*-inositol, 500 mg l^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine–HCl, 1 mg l^{-1} thiamine–HCl, 7 g l^{-1} agar (Sigma, St. Louis, MO), and 1 mg l^{-1} (4.4 μ*M*) 6-benzylaminopurine (BA), pH 5.8, and incubated at 31°C in the dark. Friable, embryogenic callus (1 g) was collected after 5–6 wk, macerated with a spatula and transferred to 35 ml liquid B1 medium in a 125 ml Erlenmeyer flask. Flasks were incubated at 25°C in the dark on a rotary shaker (125 rpm). After 1 wk, suspensions were diluted 1:1 with fresh B1 medium and cultured for another 6 d.

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Plating of cell suspension cultures. Several suspensions were combined to reduce random differences between the individual cultures and strained through 850 μm mesh. Cells (200 mg fresh weight) were layered onto a sterile filter paper disc (Whatman 3, 70 mm) by vacuum filtration and placed in a Petri plate (100 × 25 mm) with 40 ml of one of the following media: B1, B1T1 [B1 medium with 1 mg l^{-1} (2 μ*M*) 2,3,5-triiodobenzoic acid (TIBA)], B1A2, B1A0.2, or B1A0.02 [B1 medium with 2, 0.2, or 0.02 mg l^{-1} (7.6, 0.76, or 0.076 μ*M*) abscisic acid (ABA), respectively]. Plates were sealed with Parafilm and incubated at 25 ± 2°C under low light conditions (12 μmol m^{-2} s^{-1}) and a 16 h diurnal photoperiod. Cells were transferred to fresh media after 5 wk. To test the effect of osmoticum on the regeneration capacity of nonbombarded sugar beet suspension cells, cells prepared as above were kept on B1T1 medium with 44.6 g l^{-1} mannitol and 44.6 g l^{-1} sorbitol (B1T1MM) for either 4 h or 4 h + 48 h and then transferred to B1T1 medium and cultured for 5 wk. Experiments were repeated three times with five plates per replicate. Lethal concentrations of kanamycin sulfate (Km; Sigma) and paromomycin sulfate (Pr; Sigma) were determined using plated cells at antibiotic concentrations ranging from 10 to 150 mg l^{-1}.

Plasmid DNA. Transformation vectors (Fig. 1) carried the reporter gene *uidA* (*gusA*) fused to either the tobacco osmotin (Osm) or the potato proteinase inhibitor II (Pin2) gene promoter and the selectable marker gene NOS-*npt* II for Km or Pr resistance (Snyder et al., 1999). Plasmid DNA was purified using the Plasmid Maxi Kit (Qiagen Inc, Valencia, CA).

Particle bombardment. Cell suspensions were prepared as described above and placed on B1T1 medium for 24 h and then transferred to B1T1MM medium 4 h prior to bombardment. Gold particles (1.6 μm; Bio-Rad, Hercules, CA) were coated with plasmid DNA (Ingersoll et al., 1996) and bombarded under vacuum (27 in. Hg; 1 in. Hg = 3.386 kPa) using a 1350 psi rupture disc (1 psi = 6.895 kPa), 0.6 cm distance from rupture disc to macrocarrier and 11 cm microcarrier travel distance (Biolistic Particle Delivery System PDS-1000/He, Bio-Rad). After bombardment, the osmoticum concentration was gradually reduced over a period of 16–48 h (Russell et al., 1992) and filters were transferred to B1T1 medium with or without antibiotics.

Selection of transformed tissues. Bombarded cells were transferred after 2–24 d to B1T1 medium with either Km (50 or 100 mg l^{-1}) or Pr (25 mg l^{-1}) for selection of resistant cells using several different protocols (Table 2). All cultures were incubated at 25°C under low light conditions and a 16-h diurnal photoperiod for 5 wk before being transferred under cool-white lights (30 μmol m^{-2} s^{-1}). GUS (+) calluses were multiplied in B1 liquid or on B1 agar medium and then transferred to solid B1, B1T1, B1A2, and B1A0.2 medium for shoot regeneration. Calluses were subcultured every 4 wk.

Histochemical analysis of uidA *expression.* Bombarded cells on filters, pieces of each regenerated callus, or whole embryos were incubated in X-Gluc solution at 37°C overnight (Jefferson et al., 1987). Blue spots were visually tallied using a dissecting microscope. Clumps of blue cells were scored as a single transformation event.

Molecular analyses. Plant DNA was purified according to Haymes (1996). Ready To Go PCR beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) were used for polymerase chain reaction (PCR) amplification using gene-specific primers for *uidA* (forward primer, 5′-GGT CAG TCC CTT ATG TTA CG-3′; reverse primer, 5′- GTG TAG AGC ATT ACG CTG CG-3′) and *npt* II (forward primer, 5′-GAG GCT ATT CGG CTA TGA CTG-3′; reverse primer, 5′-ATC GGG AGC GGC GAT ACC GTA-3′) that amplify a 0.5 kb and a 0.7 kb fragment, respectively. After initial denaturation at 94°C for 4 min, 30 cycles of 94, 55, and 72°C for 1 min each, followed by a 72°C for 7 min extension were carried out in a thermocycler (Eppendorf Scientific, Inc., Westbury, NY). Reaction products were subjected to electrophoresis on a 1% (w/v) agarose gel.

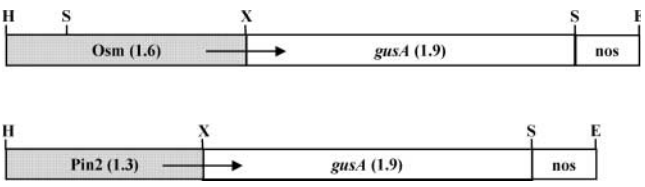

FIG. 1. Schematic representation of gene constructs used for transformation. E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xba*I.

For Southern blot hybridization analysis, 10 μg of plant DNA was digested with restriction enzymes, separated on a 0.8% (w/v) agarose gel and blotted onto a positively charged nylon membrane using 20 × SSC (0.3 *M* sodium citrate, 3 *M* sodium chloride). Blots were hybridized with a 0.54 kb *uidA*-specific probe labeled with digoxygenin-dUTP using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN). Hybridization signal generation and detection were performed using chemiluminescent substrate as described by Roche. Blots were exposed to an X-omat AR film (Kodak, Rochester, NY) for 25 min.

Results and Discussion

Culture media effect on regeneration potential of suspension cells. To establish an efficient *in vitro* system for sugar beet transformation, suspension cultures initiated from leaf callus of breeding line FC607 were cultured on five different media: B1, B1T1, B1A2, B1A0.2, and B1A0.02. Short-term suspensions that were made up of single cells and clumps of 5–20 cells were used since our preliminary results showed that the regeneration potential of these suspensions was lost after approximately 8 wk of culture. Callus grew as a confluent lawn on solid B1 and B1T1 medium after 5 wk of culture (Fig. 2*A*), whereas on ABA-containing media generally fewer than 10 callus colonies developed (data not shown). Although both somatic embryos and adventitious shoots were produced on all of the tested media (Fig. 2*A*, *B*), the greatest number

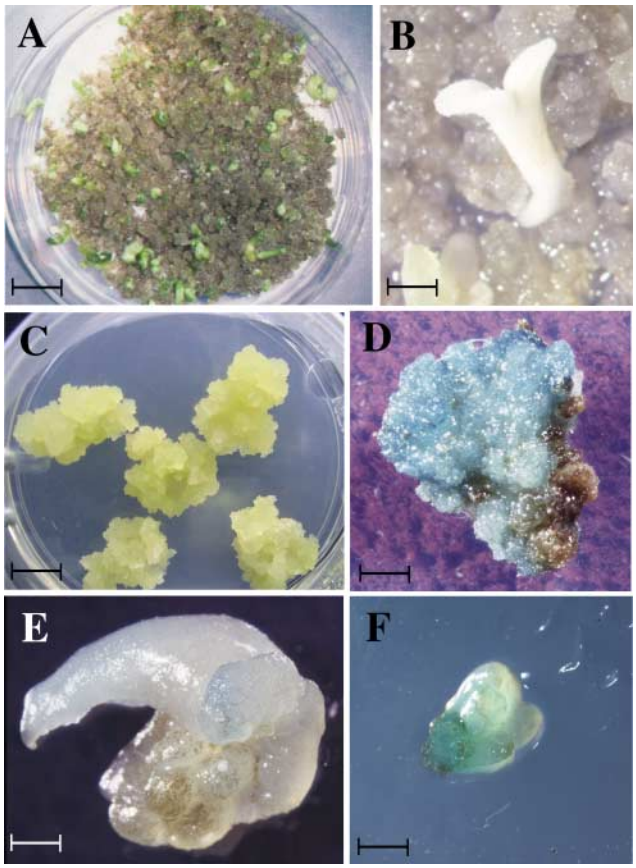

FIG. 2. *A*, Callus growth and shoot regeneration in sugar beet suspension cells on B1T1 medium at 5 wk (*bar* = 12 mm). *B*, Somatic embryo in (*A*) (*bar* = 0.9 mm). *C*, Km-resistant callus on B1 medium with 100 mg l^{-1} Km (*bar* = 11.7 mm). *D*, Histochemical GUS analysis of callus in (*C*) (*bar* = 1 mm). *E*, *F*, GUS (+) embryos (*bar* = 1 mm).

TABLE 1

EFFECT OF THE CULTURE MEDIUM ON THE REGENERATION POTENTIAL OF PLATED SUGAR BEET SUSPENSION CULTURES

Medium	Number of shoots per plate
B1	26.2 ± 5.91 a
B1T1	80.0 ± 19.89 b
B1A2	8.7 ± 3.15 a
B1A0.2	5.0 ± 1.29 a
B1A0.02	7.0 ± 0.91 a

Each value is the mean of five plates ± SE.

Entries followed by different *letters* are significantly different at $P = 0.05$ by one-way ANOVA.

of shoots regenerated on B1T1 medium (80 ± 19.9 shoots per plate; Table 1). B1T1 medium contained TIBA which has been shown to enhance sugar beet organogenesis (Tetu et al., 1987; Roussy et al., 1996) and somatic embryogenesis (Kulshreshtha and Coutts, 1997; Moghaddam et al., 2000). Cells on B1T1 medium produced up to 150 shoots per plate after 10 wk of culture.

Numerous studies have shown the beneficial effect of osmotic conditioning on transient (Walter et al., 1994) and stable transformation frequencies (Vain et al., 1993). We tested mannitol and sorbitol (0.25 *M* each) for their effect on shoot regeneration from nonbombarded suspension cultures. Neither the 4 h osmotic treatment nor the combined 4 h + 48 h treatment affected the regeneration capability of FC607 suspension cells (data not shown).

Transient and stable transformation. Plated suspension cultures were bombarded with gold particles coated with the *uidA* gene fused to the Osm or Pin2 gene promoters. These promoters were expressed at higher relative levels when compared to the constitutive CaMV 35S promoter in biolistically treated nonregenerative sugar beet cell suspensions (Ingersoll et al., 1996). Two days after bombardment, DNA delivery frequencies for both Osm-*gus* and Pin2-*gus* gene constructs ranged from 500 to 4000 *uidA* expression units (blue spots) per plate of 200 mg of bombarded callus. To evaluate the effect of a selection agent on the transient *uidA* gene expression, three bombarded filters were divided into thirds 2 d after transformation. One-third of each filter was used for the destructive β-glucuronidase (GUS) assay, and the other two were placed on B1 medium with Km (100 mg l^{-1}) and Pr (25 mg l^{-1}). The average number of *uidA*- expressing cells decreased to 0.16% of the population that initially expressed the *uidA* gene (four out of 2508) after 12 d of culture on the Km-containing medium, whereas on the Pr medium 5.46% (137 out of 2508) of the cells expressed *uidA* as compared to the initial observation.

A range of selection conditions was applied in an attempt to obtain stable transformation. In preliminary experiments, 50 mg l^{-1} of Km or 25 mg l^{-1} of Pr was determined to prevent shoot regeneration from plated sugar beet suspension cells while still allowing for growth of several callus colonies per plate (data not shown). To test more stringent selection conditions, 100 mg l^{-1} of Km was also included in these experiments. Selection was applied at 2, 7, 12, or 24 d after bombardment. A preculture period on medium without selection was crucial for successful transformation of some plant species (Fitch et al., 1990; Finer and McMullen, 1991). Regardless of the time of Km application, bombarded tissues turned dark brown during the first 2 wk on 50 or 100 mg l^{-1} Km, and shoots that had developed during the preselection period bleached. Km-resistant, GUS (+) calluses appeared on the dying tissue after two transfers to 20 mg l^{-1} Km (Table 2: Ia) or after the tissue was transferred to medium without Km (Table 2: Ib, c). If the tissues were grown for one or two more passages on the same concentrations of Km (50 or 100 mg l^{-1}, Table 2: Id–f), GUS (+) calluses developed on the dying tissue approximately 6 wk after the last transfer, presumably after the Km concentration was reduced due to degradation. The greatest number of GUS (+) calluses per bombarded plate was obtained when 50 or 100 mg l^{-1} of Km was applied 2 d after transformation for 3–5 wk, followed by reduced Km levels (0 or 20 mg l^{-1}, Table 2: Ia–c). Delay of Km selection for 7–24 d had a negative effect on recovery of stably transformed calluses (Table 2: Ie, f).

Compared to other related aminoglycosides, Pr was superior for selection of transformed cassava (Schopke et al., 1996), oat (Torbert et al., 1995), and sunflower (Escandon and Hahne, 1991). In our experiments, regardless of the duration of the preselection period, GUS (+) calluses developed 7 wk after the last transfer to Pr-containing media. We presume that these calluses, as in the case of Km selection, developed after the Pr concentration in the medium was reduced due to degradation. A relatively low number of GUS (+) calluses per plate was obtained (Table 2: IIa–c), despite

TABLE 2

INFLUENCE OF THE SELECTION PROTOCOL ON TRANSFORMATION FREQUENCIES OF BOMBARDED SUGAR BEET SUSPENSION CULTURES

Experiment no.		Selection agent	Days after bombardment to selection	Selection protocol[a]	GUS (+) calluses per plate[b]
I	a	Km	2	50/3.5 → 20/2 → 20	2.00
	b		2	100/3 → 0	1.75
	c		2	100/5 → 0	3.00
	d		2	100/3 → 100/6 → 100	0.50
	e		7	50/3 → 50/3 → 50	0.33
	f		24	50/2 → 50	0.25
II	a	Pr	2	25/3 → 25/3 → 25	0.89
	b		7	25/3 → 25/3 → 25	0.33
	c		12	25/3 → 25/3 → 25	0.40

The Osm-*gus* gene was used for all transformations, except the Pin2-*gus* was used in Ib and Id.

[a]Values indicate mg l^{-1}/wk on selection medium; *arrows* indicate transfers; after the last transfer, tissues remained on the media for up to 2 mo.

[b]Four to 12 plates were used except in Ia where only two plates were not lost due to contamination.

the higher transient expression observed on Pr- as compared to Km-containing plates.

In addition to the GUS (+) calluses, histochemical GUS staining detected seven GUS (+) embryos at different stages of development. These embryos originated from experiment Ia (one embryo), Ic (four embryos), and IIb (two embryos) (Fig. 2*E*, *F*). We believe that these embryos were stable transformants since they developed 5 (Ia), 9 (IIb) and 11 (Ic) wk after bombardment. We were not successful at regenerating transgenic plants from any of the stably transformed GUS (+) callus lines on B1, B1T1, B1A2, or B1A0.2 media.

PCR analyses of the GUS (+) callus lines revealed the expected size fragment for *uidA* (0.5 kb) and *npt* II (0.7 kb) genes (Fig. 3*A*). To confirm the integration of the *uidA* gene into the sugar beet genome, DNA of GUS (+) calluses was hybridized with the *uidA* gene (Fig. 3*B*, data not shown). One (Osm-*gus* 21; Pin2-*gus* 3) to multiple (Pin2-*gus* 1; Osm-*gus* 25) integrations of the *uidA* gene were detected in the *Xba*I digested DNA. The expected 2.8 kb Osm-*gus* and 3.2 kb Pin2-*gus* promoter-gene fragments, as well as numerous other size fragments, were detected, indicating possible rearrangements of the *uidA* gene and/or incomplete digestion of the genomic DNA (Fig. 3*B*; *Sac*I; *Hin*dIII/*Sac*I digest). The estimated *uidA* copy number was low (Osm-*gus* 21, Pin2-*gus* 3), medium (Pin2-*gus* 1), and high (Osm-*gus* 25). About 77% of the GUS (+) calluses were chimeric, showing sectors of blue color. It is possible that the calluses were of multicellular origin, consisting of a mixture of normal and transformed cells. Chimerism could also be the result of *uidA* gene silencing, but we did not see a correlation between high gene copy number that has been shown to induce gene silencing (Meyer, 1998) and the observed chimerism in GUS staining (Fig. 3*B*).

As the choice of a suitable target and a selection system are critical elements in every transformation, in this report we evaluated sugar beet suspension cultures and two selectable agents with the particle bombardment method. We found that the use of short-term suspension cultures to transform sugar beet was less time consuming and labor intensive than the particle bombardment protocol that uses embryogenic callus generated from hypocotyls (Snyder et al., 1999). A selection protocol using Km enabled recovery of a higher number of transformed calluses per plate than Pr selection. Since the GUS test is destructive, it prevented the recovery of transformed shoots from GUS (+) embryos. A different

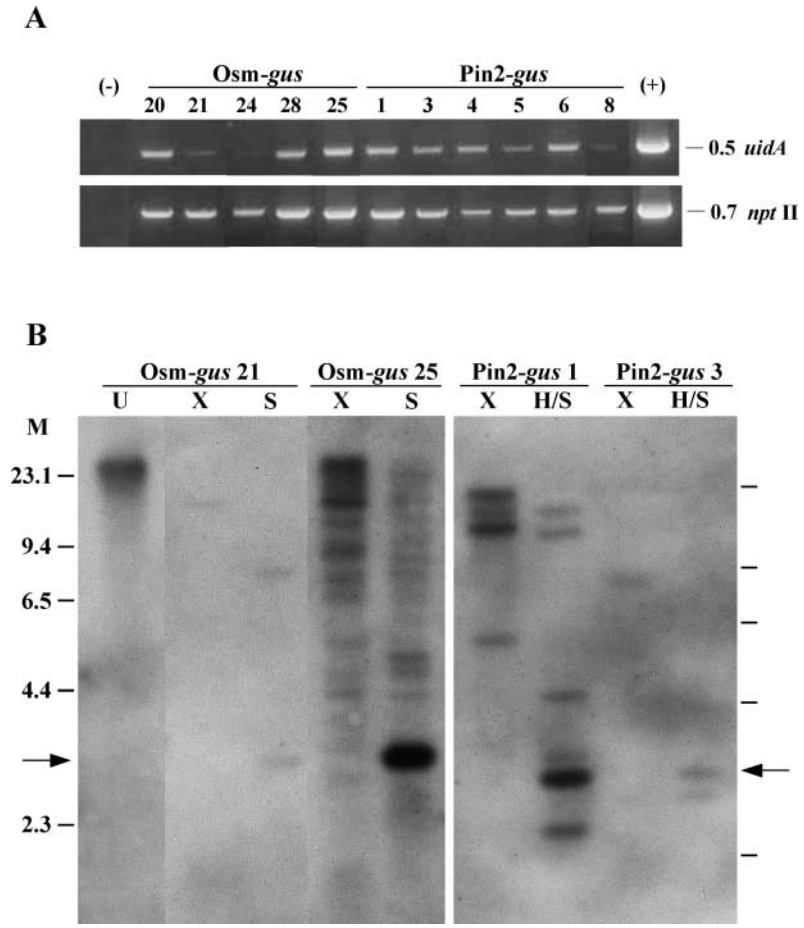

FIG. 3. *A*, PCR amplification of *uidA* (0.5 kb) and *npt* II (0.7 kb) gene fragment in independently transformed Osm-*gus* and Pin2-*gus* callus lines. (−), negative control, nontransformed callus; (+), positive control, plasmid DNA. (*B*) Southern blot hybridization with a 0.54 kb digoxygenin-labeled *uidA* gene fragment as a probe. Except for the undigested DNA (U), genomic DNA was digested with *Xba*I (X) with a single site within the promoter-gene construct to show the number of genomic integration sites of the transgene, and *Sac*I (S) or *Hin*dIII/*Sac*I (H/S) that cut at both ends of the promoter-gene construct to show the predicted fragment sizes of Osm-*gus* (2.8 kb) or Pin2-*gus* (3.2 kb) (*arrows*).

marker gene, such as the one coding for the green fluorescence protein (GFP), may be a better nondestructive alternative for visualization of transformed sugar beet tissues (Zhang et al., 2001). As we were able to initiate highly-embryogenic leaf callus from most of the breeding lines we tested (Ivic et al., 2001), using GFP as a nondestructive marker may lead to the recovery of stably transformed sugar beet.

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